Bradyrhizobium inga sp. nov., isolated from effective nodules of Inga laurina grown in Cerrado soil.

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Title

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Short title

Bradyrhizobium ingae sp. nov.

Contents category

New taxa

Subsection

Proteobacteria

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *dnaK*, *glnII*, *gyrB* *recA*, *rpoB*, *nodC* and *nifH* gene sequences of *Bradyrhizobium ingae* sp. nov. BR 10250^T are KF927043, KF927055, KF927067, KF927079, KF927061, KF927073, KF927054 and KF927085, respectively. The accession numbers for all other strains are listed in Table S2.

Abstract

Root nodule bacteria were isolated from *Inga laurina* (Sw.) Willd. growing in the Cerrado Amazon region, State of Roraima (Brazil). The 16S rRNA gene sequences of six strains (BR 10250^T, BR 10248, BR 10249, BR 10251, BR 10252 and BR 10253) isolated from the nodules showed low similarities with currently described *Bradyrhizobium* species. Phylogenetic analyses of five housekeeping genes (*dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*) revealed *Bradyrhizobium iriomotense* strain EK05^T (=LMG 24129^T) to be the closest type strain (97.4% sequence similarity or less). Chemotaxonomic data, including fatty acid profiles (with majority being $C_{16:0}$ and Summed feature 8), the slow growth rate and carbon compound utilization patterns supported the assignment of our strains to the genus *Bradyrhizobium*. Results from DNA-DNA hybridisations and physiological traits differentiated our strains from the closest validly named *Bradyrhizobium* species. Symbiosis-related genes for nodulation (*nodC*) and nitrogen fixation (*nifH*) grouped together with *B. iriomotense* strain EK05^T

and *Bradyrhizobium* strain SEMIA 6434 (used as commercial inoculant for *I. marginata* in Brazil) and TUXTLAS-10 (previously observed in Central America). Based on the data, these six strains represent a novel species for which the name *Bradyrhizobium ingae* sp. nov. (BR 10250^T = HAMBI 3600^T), is proposed.

Inga Mill. (Leguminosae, Mimosoideae), tribe *Ingeae* is considered an exclusive neotropical genus containing around 300 species, some native to the Amazon region. However, several species are also found in Mexico, Antilles and other South American countries (Possette & Rodrigues, 2010; Pennington, 1997).

The pods of this genus contain seeds covered by a white sweet pulp that is rich in minerals and is used for animal as food (Possette & Rodrigues, 2010; Pennington, 1997). In addition, some *Inga* species are used in agriculture for nitrogen input especially in alley-cropping or agroforestry systems, and also for land reclamation because the plants tolerate poorly drained, acid soils and other major growth constraints (Franco & de Faria, 1997, Romero-Alvarado *et al.*, 2002; (Kurppa *et al.*, 2010).

In general, *Inga* spp. are recognized as efficient nitrogen fixers in association with root nodule bacteria, and several countries have selected efficient inoculant strains for certain species in this genus (Franco & de Faria, 1997; Kurppa *et al.*, 2010). However, very little is known about the diversity of root nodule bacteria associated with this genus.

Previous authors have suggested that bacteria which nodulate *Inga* spp. Are part of the “cowpea miscellany” group of root nodule bacteria, because the rhizobial strains isolated from nodules also nodulate and fix nitrogen efficiently with other legumes including species of *Cajanus*, *Acacia*, *Erythrina* and *Vigna* (Allen & Allen, 1939; Grossman *et al.*, 2005). Additionally, it has been reported that slow-growing strains,

including *Bradyrhizobium* are characteristic root nodule bacteria for *Inga* spp. as for other tropical legumes (Grossman *et al.*, 2005).

During a field study in 2008, 30 root nodules were collected from *Inga laurina* (Sw.) Willd. growing in natural conditions in two sites in the Cerrado (locally known as Lavrado, State of Roraima, Brazil), including Monte Cristo Experimental Field of Embrapa Roraima and a site located in the Boa Vista city (2°50'21''N, 60° 40'32,25''W; 2°57'00''**N**, 60°42'25''W, respectively). The climate in this region is classified as Aw (Köppen) with average rainfall of 1,600 mm $year^{-1}$ and an average temperature of 27°C (Araújo, *et al.*, 2001). *I. laurina* is a common species naturally occurring in the Cerrado and other ecosystems in Brazil (Condé & Tonini, 2013; Filardi *et al.*, 2008).

To collect the nodules, adult *I. laurina* plants were located and young seedlings of *I. laurina* growing under these trees were manually uprooted. Nodules presented were collected from intact roots and transported to the laboratory. Later, the nodules were superficially disinfected (Zilli *et al.*, 2004) and individually crushed and the exudate streaked onto the YMA medium (Fred & Waksman, 1928). Following purification from single colonies, 17 isolates were obtained. All strains presented typical *Bradyrhizobium* characteristics: white colonies, alkaline reaction in medium and slow-growth. Partial 16S rRNA sequencing confirmed this observation.

For the present study, six representative strains (BR 10250^{T}, BR 10248, BR 10249, BR 10251, BR 10252 and BR 10253) were selected and subjected to a more detailed polyphasic taxonomic study, including gene sequence analysis (16S rRNA, *glnII*, *gyrB*, *recA*, *rpoB*, *dnaK*, *nodC* and *nifH*), as well as DNA-DNA relatedness, fatty acid profiles and phenotypic characterization. The strains were deposited in the Diazothrophic Microbial Culture Collection -CRB-Johanna Döbereiner- (Embrapa

Agrobiologia, Rio de Janeiro, Brazil); strain BR 10250^T, was also deposited at the Hambi Collection (http://www.helsink.fi/hambi) as HAMBI 3600^T. All strains were cultured on YMA medium at 28°C and for long-term storage the cultures were lyophilized and kept at -80°C.

For PCR, genomic DNA was prepared using the RBC Bioscience kit (cat.YGB300) and the BOX PCR analysis was performed as described previously (Versalovic *et al.*, 1994). Fingerprint analysis was performed with the BioNumerics 7.01 software package (Applied Maths, Sint-Martens Latem, Belgium) using the UPGMA algorithm and Pearson correlation index. The cluster analysis showed that the six strains grouped together with 75% similarity level in three sub-groups, indicating that they represent genetically distinct strains (Fig. S1, available in IJSEM Online).

Nearly full length sequences of the 16S rRNA gene (1318bp) were obtained for all strains using the primers and conditions described previously (Radl *et al*., 2013). Sequence alignment, alignment editing and phylogenetic analyses were performed using the MEGA5 software package (Tamura *et al.,* 2011). Phylogenetic trees were constructed using the Neighbor-joining (NJ) (Saitou & Nei, 1987) and Maximum Likelihood (ML) (Felsenstein, 1981) reconstructions. The strength of each topology was verified using 1000 bootstrap replications. The overall topologies of the phylogenetic trees obtained with the NJ and ML methods were very similar (data not shown) and the ML tree is provided (Fig 1).

The six strains formed a separate branch within the genus *Bradyrhizobium* together with *B. iriomotense* EK05^T isolated from *Entada koshunensis* (Leguminosae, Mimosoideae) in Japan (Islam e*t al.*, 2008) (Fig. 1). They shared 100% sequence similarity with each other, and 98% with other *Bradyrhizobium* type strains. We, also observed that our strains clustered together with SEMIA 6434 (BR 6610) used as a

126 commercial inoculant for *Inga marginata* in Brazil (Franco & de Faria, 1997; Menna *et*
127 *al.*, 2006) and the strain TUXTLAS-10 isolated in Mexico, which are referred to be part
128 of the "BCI Bradyrhizobium lineage" common in Central America (Parker, 2003;
129 Ormeño-Orrillo *et al*., 2012).

130 Although high similarity percentages were observed for 16S rRNA, previous
131 reports have suggested that closely related *Bradyrhizobium* species do not necessarily
132 belong to the same species (Menna *et al.*, 2009, Willems *et al.*, 2001). Therefore, Multi
133 Locus Sequence Analysis (MLSA) was performed for *dnaK* (238bp), *glnII* (537bp),
134 *gyrB* (592bp), *recA* (423bp) and *rpoB* (525bp) genes following previous reports
135 (Martens *et al*., 2008; Menna *et al*., 2009; Vinuesa *et al*., 2005). Before concatenating
136 the sequences for the genes *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*, the congruence existence
137 (tree topology) and partition homogeneity tests were evaluated (Farris, *et al*., 1994). The
138 phylogenetic tree based on the concatenated sequences of the five housekeeping genes
139 (Fig. 2) revealed that our strains belonged to a monophyletic cluster with high bootstrap
140 support (100%). Sequence similarities among our strains were 99% or 100% for all
141 investigated genes (Table S1, available in IJSEM Online). The closest type strain in the
142 16S rRNA analysis, *B. iriomotense* EK05^T, showed 97.4% or less sequence similarity
143 with strain BR 10250^T for all investigated genes (Fig. 2; Table S1; Supplementary Fig.
144 S2, Fig. S3 and Fig. S4, available in IJSEM Online). These figures also showed that our
145 strains belonged to a different group than the commercial strain SEMIA 6434 and
146 TUXTLAS-10, even though they are closely related to *B. iriomotense* EK05^T.

147 For phenotypic characterization, the strains were Gram stained and were grown
148 for 7 days on YMA at different temperatures (15, 20, 25, 28, 30, 32, and 37°C), pH
149 values (4, 5, 6, 7, 8, 9, 10 and 11) and NaCl concentrations (0.1, 0.3, 0.5, 1.0, 1.5, 2.0
150 and 2.5%). Cell motility was observed by light microscopy of a wet preparation and cell

morphology by transmission and scanning electron microscopy. Oxidase activity was detected by immersion of cells in 1% N,N,N′,N′-tetramethyl-p-phenylenediamine solution and catalase activity was determined by flooding a colony with 10% (v/v) H_2O_2 and checking for the presence of bubbles. Other biochemical tests were performed by inoculating API 20NE strips (BioMérieux, France) and Biolog GN2 microplates (Biolog Inc, CA, USA) according to the manufacturer's instructions and incubating for 8 days at 28°C. The antibiotic susceptibility tests were performed on YMA using the antibiotic Sensi-disc dispenser system (Oxoid) with bio-discs (Oxoid) containing ampicillin (10 μg and 25 μg), chloramphenicol (30 μg and 50 μg), erythromycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (10 μg), penicillin (10 μg), streptomycin (10 μg and 25 μg) and tetracycline (30 μg). The plates were incubated at 28°C and read after 10 days.

Discriminating phenotypic characteristics of our strains are given in Table 1 and the details of carbon source utilization are presented in the Supplementary Table S3, available in IJSEM Online. Our strains were able to grow between 15 and 32 °C and at a pH between 4 to 8, which are common characteristics for the genus *Bradyrhizobium*. The optimum growth was verified at 28-30°C and pH 5-7 (Table 1). All strains were resistant to erythromycin, gentamicin and neomycin and sensitive to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline. Additionally, the closest type strain EK05^T showed chloramphenicol and streptomycin resistance. Enzymatic reactions were positive for catalase, oxidase, urease and hydrolysis of esculin, and negative for nitrate reduction, tryptophan deaminase, glucose fermentation, arginine dihydrolase, hydrolysis of gelatine and β-galactosidase. The *Inga* strains differed also from EK05^T in the β-galactosidase and urease reaction (Table 1).

Whole-cell fatty acid methyl esters of strain BR 10250^T were extracted according to the MIDI protocol (http://www.microbialid.com/PDF/TechNote_101.pdf, (Delamuta *et al.*, 2013). Cultures were grown for 5 days at 28°C on YMA prior to extraction. The profiles were generated using a chromatograph Agilent model 6850 and identified using the TSBA database version 6.10 (Microbial Identification System - MIDI Inc.). The most abundant cellular fatty acids detected were $C_{16:0}$ (17.51%) and Summed Feature (SF) 8 ($C_{18:1}$ w7c) (70.78%). Moderate amounts of $C_{18:1}$ w7c 11-methyl (10.8%) and $C_{19:0}$ cyclo w8c (11.71%) were also found. The presence of $C_{16:0}$ and SF 8 supports the placement of these strains in the genus *Bradyrhizobium* (Tighe *et al.*, 2000) and revealed some differences between BR 10250^T and *B. iriomotense* EK05^T, especially the lower abundance of $C_{16:0}$ (14.7%) and higher levels of $C_{18:1}$ w7c (70.78%) (Islam *et al.*, 2008).

For DNA-DNA hybridization and for the determination of the DNA G+C content, high-molecular weight DNA was prepared as described by Pitcher *et al.* (1989). DNA-DNA hybridizations were performed using a microplate method and biotinylated probe DNA (Ezaki *et al.*, 1989). The hybridization temperature was 50°C ± 1°C. Reciprocal reactions (A x B and B x A) were performed for each DNA pair and their variation was within the limits of this method (Goris et *al.*, 1998). The DNA-DNA relatedness between BR 10250^T and the closest type strain EK05^T was 65.7%, confirming that our strains belong to a new species, since the threshold recommended is 70% (Lindström & Gyllenberg, 2007). The G+C content of DNA was determined by HPLC according to the method of Mesbah *et al.* (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilised at 37°C. The solvent was 0.02M $NH_4H_2PO_4$ (pH 4.0) with 1.5% (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* DNA were used as calibration reference and control, respectively.

The DNA G+C content of strain BR 10250^T, was 63.4 mol% (Table 1), differentiating it from the closest type strain EK05^T for which the G+C mol% was 61.2 (Islam *et al*., 2008).

Nodulation and nitrogen fixation genes are required for effective legume symbiosis, therefore *nodC* and *nifH* genes were analysed according to Laguerre *et al*., (2001) and Ueda *et al*., (1995), respectively. Phylogenetic trees were constructed as described previously and the results are given in Figs. S5 and S6 (available in IJSEM Online) for *nodC* and *nifH*, respectively. Both, *nodC* and n*ifH* gene sequences analyses clustered strain BR 10250^T in the same branch as *B. iriomotense* EK05^T, but with low similarity (Table S1, available in IJSEM Online). The maximum identity observed for BR 10250^T *nodC* sequence by BLAST search (Altschul *et al*., 1990) was 92% with a strain isolated from *Ormosia fastigiata* (Leguminosae, Papilionoideae; accession n° KF031520). The BLAST and phylogenetic analysis of *nifH* gene revealed 98% sequence similarity with strain SEMIA 6434 isolated in Brazil (Fig S5, available in IJSEM Online).

To confirm the nodulation ability of the strains investigated in this study, two glasshouse experiments were performed. In the first trial the six strains were tested on *Inga edulis*, because no viable seeds of *I. laurina*, their original host, could be found. These experiments were performed in Leonard jars containing N-free nutrient solution according to Radl *et al.* (2013). Thereafter, host plant tests were performed with strain BR 10250^T on 14 different legume species using the axenic sand-culture system described previously (Howieson *et al*., 2013). For both experiments the seeds were surface sterilized and inoculated with 1 mL of YM broth suspension containing 10^9 bacterial cells grown for 5 days at 28°C. All treatments, plus an uninoculated control, were replicated four times in a split-plot design (Howieson et al., 2013). Nodulation was evaluated 60 days and 35 days after inoculation in the first and the second experiment,

respectively. Results showed that the six strains were able to nodulate *I. edulis* (Table S4, available in IJSEM Online). Strain BR 10250^T also effectively nodulated *Arachis hypogaea*, *Macroptillium atropurpureum, Vigna radiata* and *V. unguiculata*, and formed ineffective root nodules on *Glycine max*. No nodulation was observed for *Acacia ligulata*, *Cajanus cajan*, *Crotalaria juncea*, *Lupinus angustifolius*, *Ornithopus compressus*, *Phaseolus vulgaris*, *Pisum sativum, Vicia faba* and *Vigna angularis*.

The genotypic and phenotypic data presented in this study demonstrate that the strains isolated from *Inga laurina* root nodules collected in the Cerrado of the Amazonia region represent a novel species, for which the name *Bradyrhizobium ingae* sp. nov. is proposed, with BR 10250^T (=HAMBI 3600^T) as the type strain.

Description of the *Bradyrhizobium ingae* sp. nov.

Bradyrhizobium ingae [in'gae. N.L. gen. n. ingae, of Inga, referring to the fact that the bacterium was isolated from root nodules of *Inga laurina* (Sw.) Willd].

The cells are motile with polar flagella, Gram-negative rods (approximately 1.5 x 0.6 μm), aerobic, non-spore-forming (Supplementary Fig. S7). Colonies on YMA medium are circular and translucent, and have a diameter of 1 mm within 7–8 days of incubation at 28 °C. The generation time is 9.5 h in YM broth. The pH range for growth in YMA is 4–8, with optimum growth at pH 5.0-7.0. Growth occurs between 15°C and 32°C, with optimum growth at 28-30°C. Does not grow in the presence of 0.5% (w/v) NaCl or higher. Resistance to erythromycin (30 μg), gentamicin (10 μg) and neomycin (10 μg), and sensitive to ampicillin (10 μg), chloramphenicol (50 μg), kanamycin (30 μg), streptomycin (10 μg) and tetracycline (30 μg) were observed. Positive reactions were

recorded for the utilization of the carbohydrates, D-arabitol, D-fructose, D-galactose, D-mannitol, D-mannose, D-sorbitol, L-arabinose, L-fucose, L-rhamnose, m-inositol, N-acetyl-D-glucosamine, xylitol and α-D-Glucose. Oxidase, catalase and urease were also positive, while nitrate reduction and β-galactosidase are negative. The most dominant cellular fatty acids were $C_{16:0}$ and summed feature 8 ($C_{18:1}$ w7c). DNA G+C content of the strain BR 10250^T is 63.4 mol%. The type strain BR 10250^T (=HAMBI 3600^T) was isolated from *Inga laurina* nodules collected in a Cerrado area of Amazon, from Roraima State-Brazil.

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Table 1. Different features of *Bradyrhizobium ingae* sp. nov. strains and closest related *Bradyrhizobium iriomotense* strain EK05$^{T(1)}$.

Characteristic	BR 10250^{T}	BR 10248	BR 10249	BR 10251	BR 10252	BR 10253	EK05^{T}
C source utilization							
Gentiobiose	-	-	-	-	-	-	+
m-Inositol	+	+	+	+	+	+	-
L-Rhamnose	+	+	+	+	+	+	-
Xylitol	+	+	+	+	+	+	-
Succinic acid	+	+	+	+	+	+	-
p-Hydroxyphenylacetic acid	-	-	-	-	-	-	+
Malonic acid	-	-	-	-	-	-	+
Sebacic acid	-	-	-	-	-	-	+
L-glutamic acid	-	-	-	-	-	-	+
Glycyl-L-aspartic acid	+	+	+	+	+	+	-
L-Threonine	+	+	+	+	+	+	-
D,L-Carnitine	-	-	-	-	-	-	+
Urocanic acid	-	-	-	-	-	-	+
Inosine	+	+	+	+	+	+	-
Uridine	+	+	+	+	+	+	-
Thymidine	+	+	+	+	+	+	-
L-Alaninamide	-	-	-	-	-	-	+
Enzymatic reaction							
β-galactosidase	-	-	-	-	-	-	+
Nitrate reduction	-	-	-	-	-	-	+
Antibiotic resistance							
Chloramphenicol (50 μg)	-	-	-	-	-	-	+
Penicillin (10 μg)	-	+	+	-	-	-	+
Streptomycin (10 μg)	-	-	-	-	-	-	+

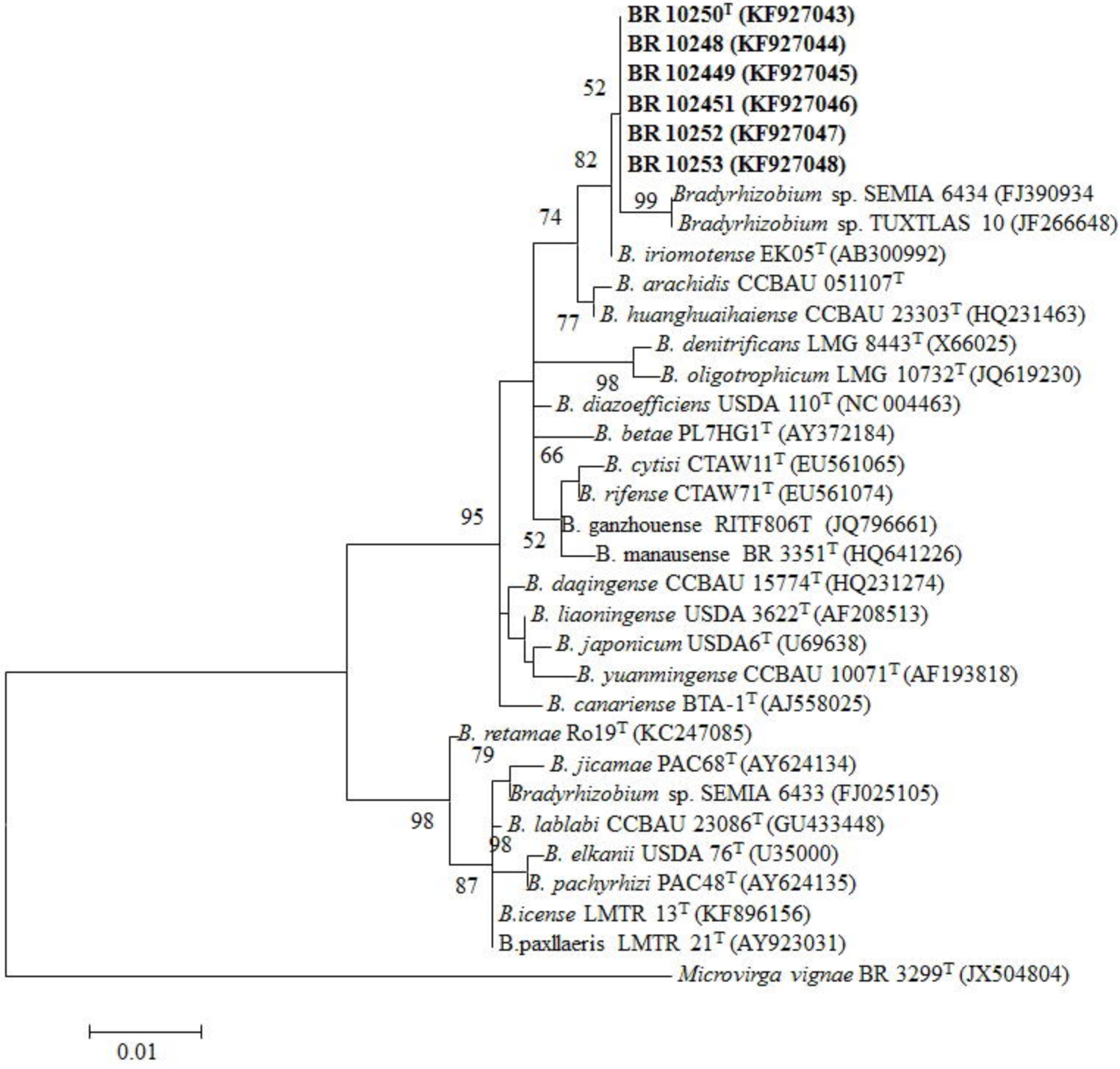
Temperature Growth range (°C)	15-32	15-32	15-32	15-32	15-32	15-32	15-32
pH growth range	4-8	4-8	4-8	4-8	4-8	4-8	4,5-9
Generation Time (h)	7.8	Nd	Nd	Nd	Nd	Nd	7-9
NaCl tolerance (%)	0.5	0.5	0.5	0.5	0.5	0.3	1.0(2)
DNA G+C content (% mol)	63.4	ND	ND	ND	ND	ND	61.2

(1) It was used the strain LMG 24129^T (formal deposit of the strain EK05^T) obtained from the LMG culture collection.

(2) Less than 1% (Islam *et al.*, 2008)

Fig. 1 - Maximum likelihood phylogeny based on 16S rRNA gene sequences showing the relationships between *Bradyrhizobium ingae* strains (shown in bold) and other members of the *Bradyrhizobium* genus. The strains SEMIA 6433 and SEMIA 6434 are commercial inoculants in Brazil for *Inga marginata*. The significance of each branch is indicated by a bootstrap value (greater than 50% showed) calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions. Sequence accession numbers of the 16S rRNA genes are presented in parenthesis.

Fig. 2. Maximum likelihood phylogeny based on concatenated *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* gene sequences showing the relationships between strains from the novel species (shown in bold) and other members of the *Bradyrhizobium* genus. The significance of each branch is indicated by a bootstrap value (greater than 50% showed) calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions.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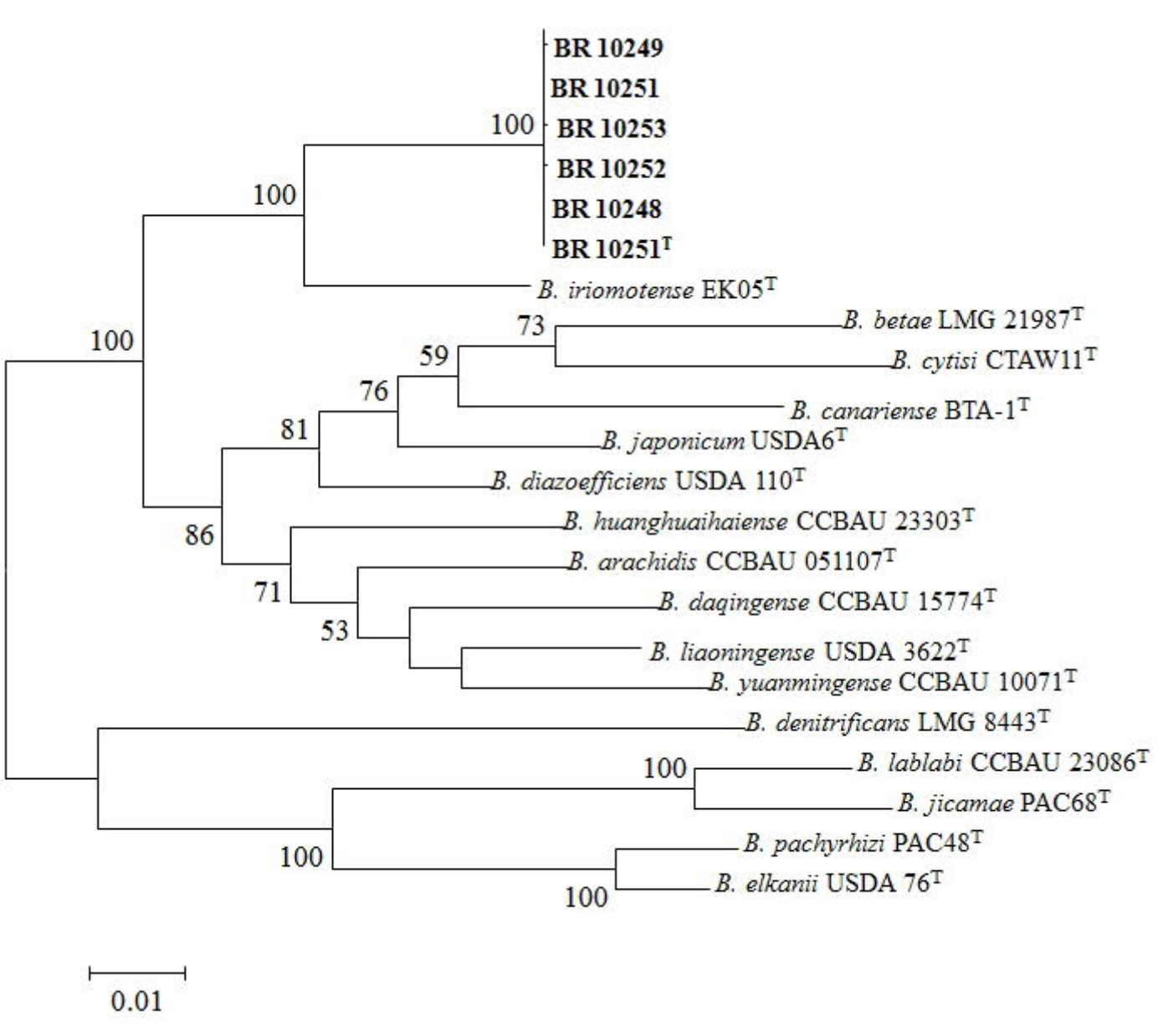
BR 10248 (KF927044)
BR 102449 (KF927045)
BR 102451 (KF927046)
BR 10252 (KF927047)
BR 10253 (KF927048)
Bradyrhizobium sp. SEMIA 6434 (FJ390934
Bradyrhizobium sp. TUXTLAS 10 (JF266648)
B. iriomotense EK05T (AB300992)
B. arachidis CCBAU 051107T
B. huanghuaihaiense CCBAU 23303T (HQ231463)
B. denitrificans LMG 8443T (X66025)
B. oligotrophicum LMG 10732T (JQ619230)
B. diazoefficiens USDA 110T (NC 004463)
B. betae PL7HG1T (AY372184)
B. cytisi CTAW11T (EU561065)
B. rifense CTAW71T (EU561074)
B. ganzhouense RITF806T (JQ796661)
B. manausense BR 3351T (HQ641226)
B. daqingense CCBAU 15774T (HQ231274)
B. liaoningense USDA 3622T (AF208513)
B. japonicum USDA6T (U69638)
B. yuanmingense CCBAU 10071T (AF193818)
B. canariense BTA-1T (AJ558025)
B. retamae Ro19T (KC247085)
B. jicamae PAC68T (AY624134)
Bradyrhizobium sp. SEMIA 6433 (FJ025105)
B. lablabi CCBAU 23086T (GU433448)
B. elkanii USDA 76T (U35000)
B. pachyrhizi PAC48T (AY624135)
B.icense LMTR 13T (KF896156)
B.paxllaeris LMTR 21T (AY923031)
Microvirga vignae BR 3299T (JX504804)
52
82
99
74
77
98
66
95
52
79
98
98
87
0.01


BR 10249
BR 10251
BR 10253
BR 10252
BR 10248
BR 10251T
B. iriomotense EK05T
B. betae LMG 21987T
B. cytisi CTAW11T
B. canariense BTA-1T
B. japonicum USDA6T
B. diazoefficiens USDA 110T
B. huanghuaihaiense CCBAU 23303T
B. arachidis CCBAU 051107T
B. daqingense CCBAU 15774T
B. liaoningense USDA 3622T
B. yuanmingense CCBAU 10071T
B. denitrificans LMG 8443T
B. lablabi CCBAU 23086T
B. jicamae PAC68T
B. pachyrhizi PAC48T
B. elkanii USDA 76T
100
100
100
73
59
76
81
86
71
53
100
100
100
0.01